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by

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This report covers the period from February 1, 1954 to November 30, 1954.

Summary

Studies have been continued on transduction by phage in Salmonella, and in a new system, in Escherichia coli. The role of the phage seems to vary from that of a passive vector to an active association with genetic material of the bacterial host. Lysogenization thus appears to be equivalent to the transduction of a particular fragment of the bacterial chromosome, which functions as the prophage.

Research on the sexual process of E. coli strain K-12 has been advanced by the development of microscopic techniques for the isolation of conjugal pairs.

New methods for the analysis of drug resistance are reported.

GENETICS OF BACTERIA

Introduction and general notes

Despite its superficial diversity, the research program in this laboratory continues to center on the theme of recombination mechanisms in bacteria. Each worker and student in the laboratory including the principal investigator is responsible for his individual problem on this theme to the extent of his own ability. Our present grants (locally administered funds; National Cancer Institute and Rockefeller Foundation) provide individual salaries, on one hand, and common expenses on the other, and there has been no attempt to segregate the work of the laboratory into separately administered projects. We have stressed a comparative approach, not only to help to generalize our conclusions on genetic mechanisms beyond a single organism, but for the stimulus that each special project receives. For example, the technique worked out last year for the pedigree analysis of clones of motile transductions in Salmonella (1) has now furnished the key to the isolation of conjugal pairs in Escherichia coli. This organization has its disadvantages when summaries are required for several lines of work at different stages of completion:

1. Lysogenicity and transduction in E. coli. [Dr. E. M. Lederberg and Mr. M. L. Morse (who is presently finishing his Ph.D. dissertation)].
2. Lysogenicity and transduction in Salmonella. [Alec Bernstein, M.D. Dr. Bernstein is on leave from the British Public Health Labor. Serv. to qualify for a Ph.D. here, probably in June].
3. Sexual behavior in various strains of E. coli. [Dr. E. M. Lederberg, Dr. Bernstein, Dr. T. C. Nelson (recently moved to Rutgers Univ.), Dr. Cavalli, Mrs. A. Cavalli, and Helen Byers (M.S., grad. stud.)].
4. Conjugation and pedigree analysis in E. coli.
5. Mechanisms of drug resistance. [Cavalli].
6. (New projects). Genetic mechanisms in actinomycetes. [S. G. Bradley, Ph.D. (Northwestern); N.R.C.-Lilly fellow. Cytoplasmic transmission and dikaryosis in yeast. [R. E. Wright, B.S. (Adelaide), M.S. (Biochemistry, Wis.) [Mr. Wright became interested in this topic while studying a biochemical problem in yeast and shifted his major accordingly]. Genetic mechanisms in Agrobacterium tumefaciens. [Mrs. D. Gosting, M.S. Mrs. Gosting's principal duties are in managing the lab. routine, and she is pursuing this problem (with collaborative help from Prof. A. J. Riker) in her spare time, when she can. She is primarily interested in the biological significance of "star forms", as a basis for more detailed genetic study of this organism.
7. (Visiting investigators). We have enjoyed two extended visits. In the spring, S. D. Rubbo (Professor of Microbiology at Melbourne; Commonwealth Fund support) spent about four months laying the groundwork for a study on yeast. Ephrussi has described cytoplasmic mutants which have lost their principal oxidative metabolism (presumably mitochondrial). We were concerned with efforts to restore the lost particles by "infection" with mitochondria, etc., from normal cells. For obvious technical reasons, if nothing else, these experiments have so far failed, but the project is continuing in Rubbo's laboratory and, indirectly, in Mr. Wright's work here.

In the fall, Dr. L. L. Cavalli (Serotherapeutic Institute, Milan, and University of Parma, Italy) spent a shorter time (with support from the Rockefeller Foundation). We had formerly enjoyed an extensive collaboration by mail, and the visit was an opportunity to solidify our continued cooperation. Experimental studies were continued on efforts to transmit the F factor by filtrates and on a new technique of indirect selection of resistant mutants.

8. Collaboration with Enzyme Institute. In the course of earlier studies related to the problem of genetic determination of enzymes, it had been found that intra-cellular lactase was not fully expressed in intact cells of *E. coli* but could be released by treating the cells with benzene or other lytic agents (2). Dr. Boris Rotman has been following up this finding. His operations are centered at the Enzyme Research Institute, but part of his salary and various materials have been administered here. He has found that under all circumstances that result in the "activation" of lactase, large amounts of RNA are released into the medium, while the enzyme may remain associated with the bacteria. There is also a general correlation between the amount of RNA that can be removed and the degree of activation. While these findings suggest that intra-cellular RNA (possibly the enzyme forming mechanism) is also regulating lactase activity, other possibilities have not been ruled out.

In the following account, I shall rely heavily on a previous report (1) and publications. These will be furnished on request. The next section summarizes progress along some of the lines titled above over the last ten months. The limited number of references to other workers is only in deference to the purpose of this report.

1. *E. coli* transduction and lysogenicity

In a number of bacteria, mechanisms have been found for the transmission of fragments of genetic material from one cell to another, either as free DNA(?) particles in extracts, or as adventitious inclusions in virus particles. This mechanism of genetic exchange, proposed to explain Griffith's early findings on "pneumococcus transformation" as well as our more recent results with *Salmonella* (3) has been called "transduction". In the *Salmonella* system every genetic marker that could be tested was found to be capable of transduction via phage, with only a slight quantitative variation from one marker to another. It was therefore concluded that the bacterial nucleus was essentially randomly broken in the course of phage lysis, and that fragments were only accidentally included in the maturing phage particle. The fragments are so small that only a few isolated examples of linkage of two markers have been found. We know very little of the localization of phage in lysogenic *Salmonella* (as compared to information on lambda in *E. coli*), but there is no present evidence to contradict this picture. It is still possible that the fragment that is included in a phage particle is related to the possible site that happened to be occupied by that phage; but phage induced from lysogenic clones of *Salmonella* has the same generalized activity as that obtained by external infection and lysis.

In *E. coli*, it had been established that the phage lambda did occupy a specific locus in the bacterial genotype (1,4). Preliminary tests showed that lambda did not mediate transduction, at least not in the generalized fashion shown in *Salmonella*. Subsequently, it has been found that lambda, obtained by UV-induction of lysogenic bacteria, will transduce a cluster of

loci (Gal₁, Gal₂, etc; concerned with galactose fermentation) which are precisely those markers that are closely linked to pro-lambda in lysogenic bacteria (5). The specificity of this transduction, together with the finding that "lytically grown" lambda was poorly competent, if at all, suggests that here is more than an accidental relationship of the bacterial fragment to the phage. This conclusion was reinforced by the discovery of derived stocks, the lambda from which will transduce Gal⁺ to Gal⁻ recipients with an efficiency of better than one per ten phage particles. The conclusion to which we are irresistibly led is that there is no objective demarcation between the genetic content of the phage and of its host bacterium, that the phage nucleus behaves in effect as if it were itself a specific fragment of the bacterial nucleus. Different genetic factors of the bacteria may show a different degree of relationship to the prophage. When this relationship is random, we have the Salmonella system (it remaining still uncertain whether inclusion of the bacterial fragment is accidental or related to the reproduction of the phage); when it is specific, but facultative (e.g., can be broken by crossing over), we have the E. coli-lambda system; when it is obligatory, we have "lysogenic conversions" which sometimes entail modifications of the bacterium that bear no obvious relationship to a state of virus infection. These are not a priori speculations, as we began these investigations with the plausible but evidently incorrect working hypothesis that phages were intra-cellular parasites inhabiting the cytoplasm. The remarkable integration of the virus with the host nucleus tells us nothing, however, of the ultimate origin of viruses, whether they stem directly from bacterial gene fragments, or whether lysogenicity is the ultimate perfection of adaptation of an exogenous parasite. Still, the essential difference between these phylogenetic hypotheses is merely whether a virus evolved by the abrupt release of a now autonomous cell fragment, or whether there was a more gradual parasitic degeneration from a free-living organism to the same terminal outcome. (Compare 6).

In addition to (perhaps in spite of) the bearing that the lambda system has on these speculative but fundamental problems, this transduction has specific advantages for experimental study. These are 1) the concurrence of sexual reproduction, allowing auxiliary cross breeding experiments; 2) the very high frequency of transduction that is possible in certain lines; 3) the fact that, in contrast to Salmonella, the immediate result of transduction is the establishment of a "heterogenic" clone in which the fragment persists side by side with its homologue in the genotype. Only as a second, later occurrence does segregation occur, with or without preceding replacement. In addition, crossing-over between the fragment and the intact genotype, without reduction, and leading to derived heterogenotes, may also occur. We can also study the interactions of closely linked genes and the effect of their relative positions in chromosome and fragment. At least two "position effects" have been found, i.e., instances where Gal⁺ genes at different loci are unable to cooperate effectively unless they are in the cis arrangement, i.e., together in either the chromosome or the fragment. The probably very large number of distinct Gal⁻ mutations (pseudoalleles?) and the technical facility of the system make this unusually favorable material for studies of gene physiology along these lines.

In addition to the role of the genetic constitution of the virus, and of other viruses in mixed infections, the host cell has been shown to play a definite part in the character of the virus released by it (7). Different lines of E. coli have been compared, and inter-crossed, with results supporting the conclusion that a definite genetic locus ("Mp") of the bacterial host differentiates the type of phage produced. This locus is distinct from Ip, the attachment site of the prophage (8).

2. Salmonella transduction

Our more recent work on Salmonella transduction has only reaffirmed the conclusions already stated (1) without advancing beyond them. It is planned to search more systematically for additional markers linked to the Fla₁-H₁ factors in order to make more decisive tests of hypotheses on the mechanism of determination of flagellar antigens (1,9). Some additional pedigrees have been made on "abortive transductions" (1,10). The number of trail-forming cells produced at a single transduction is sometimes so great as to make it unlikely that the trails simply represent unincorporated fragments. It is now suggested that they arise from the transduction of bundles of primary gene products, or the formation of the latter after a "sterile gene" has been transduced. It is not obvious how these hypotheses can be distinguished without additional linked markers.

In the course of previous studies on transduction of flagellar antigens, somatic variations were occasionally noted, but too irregularly to be rationalized as transductions. On the other hand, concerted efforts to study transduction of these antigens have failed, possibly not entirely for technical reasons (1, C. C. Spicer, unpubl.). Experiments with *S. abortus-equi* and with certain strains of *S. typhimurium* now suggest that lysogenicity per se may influence the expression of the somatic 5 antigen. The results to date are too complex to be accounted for by transduction; they more closely resemble, but still do not agree entirely with, the "lysogenic conversions" reported in *Corynebacterium diphtheriae* (11) and in other groups of *Salmonella* (12).

3. E. coli sex

Studies on diploids were summarized previously (1) and in a recent publication (13). Comparative studies are being made on the crossing behavior of a number of different lines, including some specific serotypes that have been implicated in epidemic infantile diarrhea. This rather tedious work has not yet reached a point of interest, except to note the prevalence of such lines. Previous attempts to separate the contagious "F" agent from F⁺ bacteria have been extended, still without success. In addition to the original K-12 strain, a number of other F⁺ strains have been tested to see if they would yield active millipore filtrates or extracts. To afford the maximum opportunity for recovery, the least rigorous conditions of rapid filtration were employed; one or two false alarms were clearly attributable to the penetration of intact F⁺ bacteria. F⁻ bacteria have been grown across a millipore filter (standard thickness, 0.15 mm) from an F⁺ culture without transmission of the "F agent". While new evidence (see below) supports the contention that direct cellular contact is required, experiments are in progress to test the permeability of special, thin filters (only .01 mm thickness, cf. 14).

4. E. coli pedigree analysis

It was mentioned previously (1) that single cell isolation methods (15, and micromanipulation) were being applied to the search for xygotic processes in *E. coli*, which have so far eluded all but genetic screening methods. By the use of morphologically distinct strains, one actively motile and slimmer (and Hfr), the other non-motile and plumper (and F⁻), it has been possible to recognize and isolate conjugal pairs. In non-motile cultures, these are almost impossible to recognize and disentangle, especially as they fall through the drop and the cell masses adhere to the interfaces. Now, the pairs are readily visualized at low power by virtue of their characteristic disturbed motion. The motility also facilitates clean isolations. The morphology of the side-to-side or side-to-tip connection between the conjugants has not yet been clarified, but is being sought by electron microscopy. The pairs swim about for an hour or two and then disjoin. Usually each exconjugant generates a viable clone; genetic recombinants

have been found in, and only in, the F- exconjugant clones. That is, the Hfr exconjugant retains its identity, while the F- mate throws a mixed clone containing both the parental F- genotype, and new combinations. The incidence of exconjugant clones containing detected recombinants has been 20-30% of the pairs. The frequency of primary zygotes is probably higher, owing to losses from inviability of subclones, interrupted conjugations and zygotes that do not form detected recombinants.

Under the same conditions, the incidence of recombinants in the whole F- population is under 1%; the pairs are frequent enough to account for the observed frequency of recombination. All in all, there can be little doubt of the pertinence of these pairs to the sexual process. A limited number of clonal pedigrees, for two to five or six generations, have been made of the exconjugants. Many more are needed, but it is apparent that a single nucleus from the Hfr parent fertilizes the F- cell to form one diploid zygote, while several unfertilized nuclei of the multinucleate F- and Hfr cells persist. The diploid zygote nucleus is immediately reduced, but it requires several cell generations to sort out the various heterokaryotic combinations of parental and recombinant nuclei. As in virtually all other crosses so far reported, an elimination of a chromosome segment hinders the appearance of markers from the Hfr parent in the sexual progeny. Previous data from diploids have shown that this elimination is post-zygotic, i.e., that the gamete nucleus was intact. Although the segregations are thus strongly biased in favor of the markers from the F- parent, each of the several markers from the Hfr parent (except the motility trait which may well be polygenic) has appeared in some sexual progeny. If, as is done with other microbes in stretching the concepts of sexual differentiation, we base our definitions on gamete mobility, we would be justified in describing Hfr cultures as male (more accurately as andric hermaphrodite) and the F- as female, in accordance with speculations that had been previously advanced on the basis of differential sterilization with streptomycin (16).

In agreement with observations on the occurrence of genetic recombination, pairs have been found abundantly in Hfr x F- mixtures, less regularly in Hfr x Hfr, and not at all in F- x F-. However, although the recombination frequency is very low, pairs have also been seen in F+ x F- cultures. Isolations from these pairs have not generated detectable recombinants, but they are associated with a high incidence of conversion from F- to F+ of the F- exconjugant. I also have the impression that these pairs are less durable than in Hfr x F-. These observations suggest that the mechanism of F conversion is also based on a cell to cell contact which still leaves the material basis of the phenomenon in obscurity.

5. Drug resistance

Previous studies might have been expected to be conclusive, but apparently there is still considerable confusion over experimental criteria for preadaptive mutation to drug resistance, i.e., for the restricted role of a drug as a selective agent in all cases sufficiently studied to date. Dr. Cavalli's visit created an opportunity to clean up some loose ends of Luria and Delbruck's early statistical demonstration (17), namely to show that the fluctuation they found in numbers of mutants from one culture to another is heritable to a second generation of cultures, and cannot possibly be accounted for by uncontrolled environmental differences as Hinshelwood has proposed. In addition, a modified technique of indirect selection was successfully worked out. This had been accomplished previously (18) with the help of a method called replica plating which, while convenient, does not allow exact measurement of inoculum and clone sizes on agar plates. It was reasoned that if, by spontaneous mutation, a bacterial culture of 10^9 cells/ml

contained, say, 10^2 resistant mutants per ml, then the ratio of resistant mutants to total, now $1:10^7$, could be increased ten-fold by taking samples of 10^{-3} ml as inocula for fresh cultures. On the average, these inocula will contain 10^6 cells each, and 0.1 resistant, that is to say, one in ten will have a single resistant. This particular culture will therefore have a ratio of resistants: total of $1:10^6$, and can be detected by quantitative assays after these new cultures have grown up. The same environment schedule can be repeated as often as needed, until pure resistant cultures, never having themselves been in contact with the drug, are achieved. The regimen thus outlined was quite successful for streptomycin resistance in *E. coli*, though the later stages of enrichment were somewhat hindered by selective disadvantage of the resistants in the absence of streptomycin, and by other second order effects.

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